Archives of Insect Biochemistry and Physiology 58:69–83 (2005)

Micro-Injection of Lygus Salivary Gland Proteins to Simulate Feeding Damage in Alfalfa and Cotton Flowers

Kenneth A. Shackel,[1] Maria de la Paz Celorio-Mancera,[1] Hamid Ahmadi,[1] L. Carl Greve,[1] Larry R. Teuber,[2] Elaine A. Backus,[3] and John M. Labavitch[1*]

Alfalfa and cotton flowers were pierced with small glass capillaries of an overall size and shape similar to that of Lygus stylets, and injected with small quantities (6 to 100 nL) of solutions that contained Lygus salivary enzymes. Crude and partially purified protein solutions from Lygus heads and isolated salivary glands showed substantial polygalacturonase (PG) activity, as has been previously reported. Following injection with both crude and partially purified protein solutions, as well as with pure fungal and bacterial PGs, flowers of both alfalfa and cotton exhibited damage similar to that caused by Lygus feeding. Injection with the same volume of a buffer control as well as a buffer control containing BSA at a comparable protein concentration (approximately 6 μg/mL) showed no symptoms. These results are consistent with a previously suggested hypothesis that the extensive tissue damage caused by Lygus feeding is primarily due to the action of the PG enzyme on the host tissue, rather than to mechanical damage caused by the insect stylet. Substantial genotypic variation for a PG inhibiting protein (PGIP) exists in alfalfa and cotton. We, therefore, suggest that breeding and selection for increased native PGIP levels, or transformation with genes encoding PGIP from other plant species, may be of value in obtaining alfalfa and cotton varieties that are more resistant to Lygus feeding damage. Arch. Insect Biochem. Physiol. 58:69–83, 2005.

KEYWORDS: crop protection; flower; pectin; polygalacturonase; polygalacturonase-inhibiting protein

INTRODUCTION

Lygus bugs (Hemiptera:Heteroptera:Miridae) are a serious pest of many agricultural crops, including legumes, cereals, cotton, fruits, vegetables, and canola (e.g., Carlson, 1940; Holopainen, 1986; Layton, 2000; Williams and Tugwell, 2000). The bugs feed on various plant tissues using piercing and sucking mouthparts. During stylet penetration (probing), saliva (containing many enzymes and amino acids) from the bug is introduced into the target tissues in a "lacerate and flush" (Taylor and Miles, 1994) action. Damage is manifested as tissue necrosis, distortion and abscission of fruits, growth retardation, and discoloration. For many years, the general consensus was that feeding caused only physical damage, but Strong and Kruitwagen (1968) demonstrated the presence of a potent PG in lygus salivary glands, and Strong (1970), noting that tissue maceration that accompanied insect feeding resembled that caused by incubation of excised salivary glands with plant tissues, concluded that the principal damage caused by bug feeding was due to the action of this sali-

[1]Pomology Department, University of California, Davis

[2]Agronomy and Range Science Department, University of California, Davis

[3]USDA Agricultural Research Service, Exotic and Invasive Diseases and Pests, Parlier, California

Paper presented at the 51st Annual Meeting of the Entomological Society of America, October 2003. Symposium entitled Insect Saliva: An Integrative Approach.

Abbreviations used: BSA: bovine serum albumin; HPE: head-pronutum extract; PAGE: polyacrylamide gel electrophoresis; PG: polygalacturonase; PGIP: polygalacturonase-inhibiting protein; SEC: size exclusion chromatography; SL_{50}: treatment level at which 50% of the treated plants show symptoms.

*Correspondence to: John M. Labavitch, Pomology Department, University of California, Davis, CA 95616. E-mail: jmlabavitch@ucdavis.edu

DOI: 10.1002/arch.20033
Published online in Wiley InterScience (www.interscience.wiley.com)

vary PG. More recently, Cohen and Wheeler (1998) also concluded that "there is maceration or cell wall destruction that appears to be caused directly by the saliva. The damage is clearly more extensive than could be accomplished by direct laceration by stylets." However, there has been no direct experimental evidence that substantial tissue damage will occur in the absence of stylet laceration, and hence the issue remains a matter of interpretation.

There is a substantial array of plant polysaccharide digestive enzymes in the saliva of many insects. This includes activities that hydrolyze glycosidic and ester linkages in the acidic pectin polysaccharides (e.g., pectinesterase and PG) as well as linkages in the variety of neutral glycans found in the so-called hemicellulosic cell wall polysaccharides (e.g., *endo*-1,4-β-glucanase [often called cellulase] and *endo*-1,4-β-xylanse) as well as others (see Strong, 1970; Miles, 1999). We have found that enzyme activities in the saliva of *Lygus* are similar to those reported by other researchers for the salivary enzymes of aphids. This is interesting, because the approaches that these two taxa use in feeding and the extent of the damage that results from probing and ingesting are quite different (Backus, 1988). There is debate about whether the salivary enzymes of aphids facilitate probing and placement of stylets (Miles, 1999). In contrast, the enzymes of heteropterans like the lygus bug more clearly appear to function in extra-oral digestion (Cohen and Wheeler, 1998), as well as to reduce feeding impediments posed by intact cell wall polymers (e.g., Tjallingii and Hegen-Esh, 1993). Substantial plasticity also occurs in salivary enzyme composition, apparently dependent on the sensing of the feeding substrate (Campbell and Dreyer, 1990; Zeng and Cohen, 2001).

PG is a plant cell wall–digesting enzyme commonly identified in insect watery saliva (Agblor et al., 1994; Agusti and Cohen, 2000; Cherqui and Tjallingii, 2000; Cohen and Wheeler,1998; Strong and Kruitwagen, 1968; Shen et al., 1995). Insect PGs have been purified by several groups (Agusti and Cohen, 2000; Cherqui and Tjallingii, 2000; Cohen and Wheeler, 1998; Shen et al., 1995). PG is a pectin hydrolase, specifically hydrolyzing glycosidic linkages between adjacent, unsubstituted galacturonic acid (GalA) residues in the α-1,4-linked homogalacturonan backbones of simple pectin polymers (Cook et al., 1999). Depending on the specific nature of the PG, the products of enzyme action will be oligosaccharides or monosaccharides. Because pectins are structural polysaccharides of plant cell walls, the result of insect PG action will be, at the minimum, a weakening of the defensive barrier provided by the cell wall. It is not known whether insects can use the GalA carbohydrate derived from pectin backbone digestion for their own energy metabolism.

Substantial attention has been focused on a group of plant proteins (the PGIPs) that selectively inhibit the PGs of phytopathogenic fungi (DeLorenzo and Ferrari, 2002). A variety of crop plants have been shown to have PGIP-encoding genes (Stotz et al., 2000), and often there are small families of PGIP genes, each encoding a protein with a different pattern of selective inhibition of PGs. Transgenic expression of a pear fruit PGIP-encoding gene in tomato leads to a suppression of symptoms caused by the grey mold pathogen, *Botrytis cinerea* (Powell et al., 2000). If lygus bug PG is a key factor in the crop damage the insect causes, then PGIPs might contribute to a reduction of that damage. This report describes the novel use of a microinjection system for experimentally determining the relative roles of physical damage and the presence of putative digestive enzymes in the overall damage expressed when Lygus feeds on flowers of alfalfa and cotton, and also suggests the possibility that manipulation of crop PGIP content may be an effective approach to mitigate damage caused by PG-containing insect saliva.

MATERIALS AND METHODS

Insect Supply (Field Collection and Sorting)

Lygus bugs were collected in alfalfa fields located at UC Davis (Agronomy and Range Science and Animal Science experimental fields) and at the University of California Kearney Research and Extension Center (Parlier, CA) using an entomologi-

cal sweep net. Following transport to the laboratory, only live insects were used for isolation of salivary glands. Insects used for the preparation of head-pronotum protein extracts (HPE) were held in a freezer for 24 h (–40°C) and sorting of the Lygus bugs was done immediately prior to excision of their heads and homogenization.

Enzyme Extraction and Assay

Head-pronotum extracts. Lygus bugs were sorted from the frozen, collected sample; a scalpel was used to separate the head from the rest of the body with a cut between the pronotum and scutellum. For every 0.3 g of heads, 10 mL of PG extraction buffer (0.1 M sodium acetate, pH 5, 1 M NaCl, 5 mM 2-mercaptoethanol) was added and a Polytron blender was used to homogenize the tissue. The homogenized suspension was centrifuged at 4°C for 15 min at 16,000*g* (Sorvall SS-34 rotor). The supernatant was decanted and stored at –4°C.

Enzyme assays. The PG activity of the extract was tested using a radial diffusion assay (Taylor and Secor, 1988) by placing 15 µL of extract in a well created in an agarose (Low EEO, Electrophoresis grade, Fischer Scientific, Fair Lawn, NJ) sheet containing polygalacturonic acid (Sigma-Aldrich) as substrate. The enzyme diffuses radially from the well into the agarose and digests the substrate. The PG activity is then visualized by staining the agarose sheet with ruthenium red (Aldrich). Ruthenium red binds efficiently to the intact polymer. Non-dyed areas (i.e., areas cleared of substrate) represent the activity of the PG; therefore, the area cleared is proportional to the enzymatic activity. A 15-µL aliquot of HPE yielded, on average, a cleared area of 278 mm^2 in the radial diffusion assay (see below).

Assays of cellulase, protease, amylase activity assay were performed as above but the substrates dissolved in the buffered agarose were carboxymethylcellulose, BSA, and starch (all substrates from Sigma-Aldrich), respectively. Substrate digestion was then visualized using Congo red (Sigma-Aldrich, for cellulase), Coomassie Brilliant blue (for protease; Bio-Rad, Richmond, CA), and I_2-KI solution (amylase).

Isolation of salivary glands. Salivary glands were isolated using the following techniques. Individual insects were immobilized in a small, partially melted wax droplet, dorsum-down. The legs were removed and the end of the abdomen was pulled outwards with forceps, removing the digestive tract and the reproductive system. The lateral edges of the abdomen and thorax were then cut along the body with iridectomy scissors and the thoracic and abdomenal sclerites were removed from the rest of the body. Approximately 30 pairs of glands were ground with an Eppendorf pestle in 1 mL of PG extraction buffer. The resulting slurry was then centrifuged in an Eppendorf microfuge (5 min, 16,000*g*). The salivary gland extract (supernatant) was stored at –40°C. On average, a 15-µL aliquot of the salivary gland extract gave a cleared zone of 251 mm^2 in the PG radial diffusion assay.

Partial Purification of Lygus Bug PG

The Lygus HPE was subjected to various protein purification techniques to obtain a partially purified PG. Proteins in the extract were sequentially precipitated with ammonium sulfate at 0–45, 45–65, 65–85, and 85–100% saturation. Proteins precipitated at each ammonium sulfate concentration were collected by centrifugation (15,000*g*, 10 min). The resulting pellets were suspended in 100 mM Tris-HCL, pH 7.0, and then dialyzed against the same buffer (100 volumes, 3 changes of buffer). The sample collected in the 65–85% of saturation cut contained the greatest PG activity and was used for size exclusion chromatography (SEC). The dialyzed protein sample was subjected to SEC in Sephacryl S-200 (3 × 30 cm column, eluted with Tris-HCl buffer). Fractions (2.7 mL) were collected and assayed for PG, amylase, protease, and β-1,4-glucanase using the radial diffusion assays described. PG activity was identified in several groups of fractions, indicating the occurrence of PG proteins of different molecular size. A peak of activity eluting near the S-200 column's void volume was essentially free of contaminating amylase, protease, and glucanase. The fractions containing this high molecular weight PG were pooled and concen-

trated by dialysis against polyethylene glycol compound (MW 15,000–20,000). The SEC-purified protein concentrate was then subjected to native PAGE analysis using 6% acryamide gels and the buffer system described by Laemmli (1970), absent the SDS and mercaptoethanol, using a BioRad MiniProtean electrophoresis cell. After electrophoresis, the outer lane from each side of the gel was removed and stained with Coomassie blue. This revealed that the fraction contained several resolved protein bands and a band indicating a substantial amount of protein that had just entered the resolving gel. The remainder (center section) of the gel was cut horizontally to isolate the band that had just entered the resolving gel, two lightly stained bands that ran together, just ahead of the slow-moving, high molecular weight band, as well as other bands as separate slices of gel. Each of these slices was homogenized in Tris buffer and then filtered through Whatman GF/C glass fiber paper.

PAGE "Activity Gel" Analysis of Lygus Bug PG Isoforms

A modification of the approach used by Shen et al. (1995) was used to characterize the variety of proteins in the Lygus bug HPE that had PG activity. Aliquots (60 µL) of the extract were placed in the wells of a 2-mm-thick, 18 × 16 cm polyacrylamide gel (Hoeffer SE410) and subjected to PAGE. The buffer system used was that of Laemmli (1970), however mercaptoethanol was omitted from the sample buffer and pre-run boiling of the sample was only 2 min. After electrophoresis, the gel was gently shaken with 4% Triton X-100 for 4 h and rinsed with distilled water briefly (3×). The SDS-free gel was then incubated in the polygalacturonic acid substrate (0.2% in Tris-HCl, pH 7) and incubated with gentle shaking for an additional 4 h at room temperature. Following incubation, the gel was stained in ruthenium red overnight, destained by gentle shaking in DI water (3 changes). As with the radial diffusion assay of PG, clear zones reveal the location to which the bands of PG have been electrophoresed. While in this report the technique has been used to describe the size distribution of Lygus salivary PG proteins, we anticipate using a preparative scaling of the technique to facilitate the purification of separate Lygus PG species.

Assay of PGIP Activity Directed Against Lygus Bug PG

The PG inhibitory activity of cotton and alfalfa leaf extracts was tested using a modification of the PG assay described above. Parallel assays of PG preparations were performed. In one set, a given amount of PG preparation was diluted with an equal volume of 100 mM acetate buffer before addition to wells in the polygalacturonic acid-containing agarose sheet. In the second set, the PG was mixed with an equal volume of protein extract from cotton or alfalfa leaves prior to addition to the wells. After incubation and staining with ruthenium red, a comparison of the zones cleared of substrate indicates the relative PG inhibition by proteins in the extract (Fig. 1).

Injections

Glass capillaries with tips of an overall size and shape similar to that of lygus stylets (cylindrical/conical, with a 20–30 µm outer diameter at 2 mm of length) were produced using a micropipette puller (Koph model 750) and opened in a jet stream of suspended Buehler Micro-polish (0.05 micron gamma alumina) as described by Shackel et al. (1987). Capillaries were filled with silicone oil, attached to a micro-pressure probe, and loaded with about 6 nL of a test solution for alfalfa florets (which were typically about 1 mm in overall size, see below), and about 300 nL for cotton flowers (typically about 5 mm in overall size). Tips were grossly positioned under a microscope using a mechanical micromanipulator (Leica, Germany), and a motorized piezo-electric manipulator (Stoelting, IL) was used to advance the capillary tip into the tissue and to measure the depth of penetration below the tissue surface (Fig. 2). The micro-pressure probe is a device designed to create a pressure on the oil contained within a glass capillary in order to measure cell turgor pressure. An internal pressure of maximally about 1 MPa (150 psi) can also

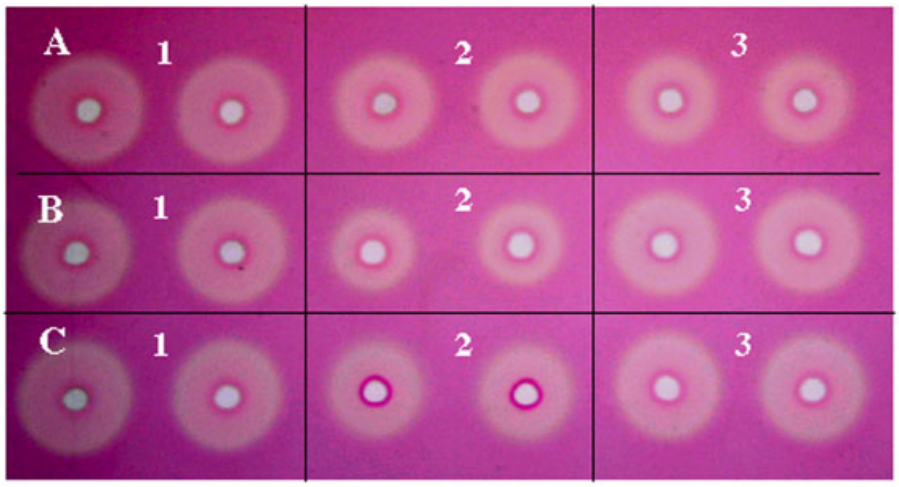

Fig. 1. Radial diffusion assay for PG and PGIP activity determination. **Row A:** Decreasing clear zones as an initial Lygus PG preparation (A.1) is diluted to 0.5 (A.2) and then 0.25 (A.3) its original concentration. If protein containing PGIP is mixed with PG, PGIP activity is shown by reduction in cleared zone diameter. **Row B:** B.1 shows no reduction after mixing of protein from a very low PGIP alfalfa with the PG shown in A.1. B.2 shows the impact of the same amount of protein from the same alfalfa line that has been transformed to the pear PGIP-gene (the clear zone area suggested inhibition of the PG of A.1 by >80%). B.3 shows the same test with a transformed line showing no enhancement of anti-Lygus PG inhibitory activity. **Row C:** The same PG activity (C.1) as in A.1 and the A.1 amount of PG mixed with proteins extracted from a cotton line with high PGIP content (C.2) or a line with low PGIP content (C.3).

be used to inject test solutions into plant tissue. Injections were performed into the stem (peduncle) at the bases of alfalfa inflorescences, into the center of developing alfalfa florets, and into various positions on developing individual cotton flowers (see below). The injection protocol was to first position the tip of the oil-filled micro-capillary inside a droplet of solution that was suspended at the end of a 50-µL syringe needle. Solution was drawn into the micro-capillary by setting a slightly negative pressure (about –0.05 MPa) in the oil, readjusting the pressure to about 0.0 MPa after the desired volume of solution had entered. Solution volume was estimated using the measured distance

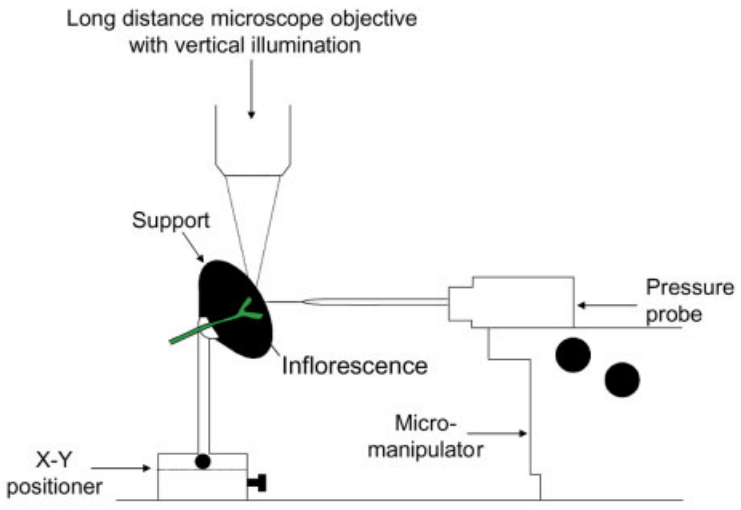

Fig. 2. Diagram of the micro-pressure probe system used to simulate *Lygus* feeding damage by micro-injection of enzyme preparations or other fluids.

from the tip to the oil/solution boundary of any given micro-capillary, based on an estimate of the internal dimensions of the micro-capillary. The internal dimension estimate was based on the average dimensions of 5 micro-capillaries that were pulled to represent a range of patterns in glass taper. The internal volume was also estimated independently for one of these micro-capillaries by measuring the diameters of spherical water drops discharged into immersion oil from different quantities of water within the micro-capillary. The size of the glass micro-capillaries was relatively uniform up to a distance of about 2 mm, giving an uncertainty of about ±2 nL for the 6-nL injections, but was more variable with distance from the tip, giving an uncertainty of about ±100 nL for the 300-nL injections.

The tissue was penetrated to the desired depth, and the pressure in the oil was increased until a progressive forward movement of the oil/solution boundary indicated that the solution was being injected. Required injection pressures ranged from about 0.1 to 1.0 Mpa, but in about 25% of cases, a 1 MPa pressure was reached without causing injection, indicating that the tip was either blocked or plugged, and in these cases the pressure was reduced to 0 MPa, the micro-capillary was advanced an additional 20–40 μm, and the process repeated until either a successful injection had occurred at the selected site (maximum 3 repeats), or a new floret (alfalfa) or second site on the same flower (cotton) was attempted with the same or a new micro-capillary. These cases probably represented a blockage of the tip rather than a plugging, because it was usually possible to cause solution and oil flow out of the tip if it was removed from the tissue. Typically, one capillary could be used for about 3 sequential injections. Once solution had been successfully injected, the pressure was returned to 0.0 MPa and/or the capillary was simply withdrawn from the tissue. In about 25% of the cases in alfalfa, and in cotton injected at the staminal column (see below), a small amount of the injected liquid would appear as a droplet on the flower surface during injection, typically near the apex of the flower. Presumably, this was the result of injecting solution into a space that communicated with the zone between the carpel and petals.

A number of protein preparations were used for the micro-injection experiment. In each case, we used a control of the sodium acetate buffer (pH 5.0) or bovine serum albumin (BSA) at a protein concentration in acetate buffer comparable to that of the PG-containing preparations used in the same experiment. Pure PGs from pathogenic fungi (*Aspergillus niger* and *Fusarium moniliforme*) and bacteria (*Pseudomonas syringae*) were also used in some injection experiments (pure fungal and bacterial PGs were provided by Dr. C. Bergmann of the Complex Carbohydrate Research Center, University of Georgia). These were diluted in buffer prior to use to the point where they gave clear zones in the radial diffusion assay comparable to those made using other PG preparations. Injections were also performed using HPE, a highly purified PG from the HPE, and the total protein extract from isolated salivary glands. The amount of PG that was injected was based on activity as measured in the radial diffusion assay, because the protein content had little relationship to the amount of PG, due to the presence of many other co-extracted proteins. The one exception was the highly, but likely incompletely purified, PG from HPE. In that case, the final protein concentration in acetate buffer was 6 μg/mL.

Plant Material Used for Injections and Injection Symptom Evaluation

Alfalfa. Crowns of alfalfa (*Medicago sativa* L.) clones were planted in 3-L plastic pots in a 1:1:1 sand/soil/Perlite mixture. Genetic background of these clones traces to the cultivar "Moapa 69" (M-69-10) and an advanced selection derived from the cultivar "UC-Impalo-WF" for the UC selections UC-2705-211 and UC-2705-177. Plants were irrigated daily by an automated irrigation system, and grown under greenhouse conditions of about 20°C and natural light, supplemented to a day length of 16 h. Plants were trimmed to the crown every 6–8 weeks and typically began flowering after about 3 weeks of vegetative growth. Plants were irrigated

and then brought to the lab for injection. An inflorescence with unopened florets that were 0.9–1.4 mm in maximum diameter was gently held in place with flexible wire and/or magnets on a support under the injection microscope (Fig. 2). The intended injection depth was the center of the floret, so the initial penetration was to about 45% of the measured floret diameter. Following injection, inflorescences were tagged and the plants returned to the greenhouse. Inflorescences were examined visually in the greenhouse for injury symptoms after 7–10 days. In some cases, inflorescences were taken to the lab and dissected.

Cotton. Seeds of cotton (*Gossypium hirsutum* L. cv "Maxxa") were planted in 3-L pots in a 1:1:1 mixture of sand/soil/Perlite and grown under the same greenhouse conditions as for alfalfa (above). After about 30 days, small flowers had formed and only the first-formed flowers on a plant (i.e., within 21 days of the appearance of the first flower) were used for injections. As for alfalfa, cotton plants were brought into laboratory conditions and the flowers positioned under the injection microscope (Fig. 2). Injections were targeted toward either the staminal column tissue at two depths below the surface of the flower (after removing the bracts) of about 1,400 and 2,200 μm, or the region of the ovary/receptacle tissue at a depth of about 2,200 μm (see results). Before injection, flower size was measured at the widest part of the flower after removing the bracts, and various sizes of cotton flowers were used: small (3.0–4.5 mm in overall diameter), medium (4.5–6.5 mm), and large (6.5–9.0 mm). Following injections, plants were returned to the greenhouse. Injected flowers were examined after various periods of time between 1 and 10 days post-injection for both internal (destructive sampling) and external symptoms. Internal symptoms were evaluated by vertically cutting flowers in half with a razor, as nearly as possible in the plane containing the injection site, and the halves were examined using a stereo microscope and in some cases photographed. Cotton symptoms were expressed on a scale of 0 (no symptoms, normal flower development) to 4 (abscised or extensive internal and external tissue browning) and the effects of the different targeting treatments were evaluated statistically using the GLM procedure of SAS (V8/windows, Cary, NC).

Screening of Alfalfa and Cotton for Anti-Lygus Bug PG, PGIP Activity: Extraction of Leaf Proteins

Alfalfa terminal leaf tissue (0.30 g) of approximately 40-day-old field-grown plants, just prior to flowering, was placed in a 1.5 mL Eppendorf tube and homogenized in 1 mL of extraction buffer (0.1 M sodium acetate, pH 5, 1 M NaCl, 5 mM 2-mercaptoethanol). The homogenate was centrifuged for 5 min at 13,000*g* in an Eppendorf microcentrifuge. The supernatant was recovered and stored at 4°C. Tissue discs 8 mm in diameter were cut from young cotton leaves (approximately 12 cm wide). Five discs (approximately 0.3 g) were placed in 1.5-mL Eppendorf tubes and ground in 1 mL of extraction buffer (as above) containing 5% w/v polyvinylpolypyrrolidone. The homogenate was centrifuged and the supernatant (protein extract) was handled as above.

RESULTS

Partial Purification of Lygus Bug PG

The mixture of proteins in the Lygus HPE was subjected to serial ammonium sulfate precipitation and the fraction with the greatest PG activity was then subjected to SEC. While several peaks of PG activity were identified by assays of collected fractions, fractions representing proteins eluting just after the S-200 column's V_o (i.e., representing proteins of relatively high molecular weight) were free of contaminating amylase, glucanase, and protease activities. Native PAGE separation of these proteins in a 6% acrylamide gel followed by protein staining revealed several protein bands (Fig. 3A). Sectors representing these bands were eluted from the unstained, central portion of the gel and assayed for PG. The two closely running bands (arrow in Fig. 3A, expanded view) that migrated just ahead of a substantial protein band that had just entered the resolving gel contained PG activity that was des-

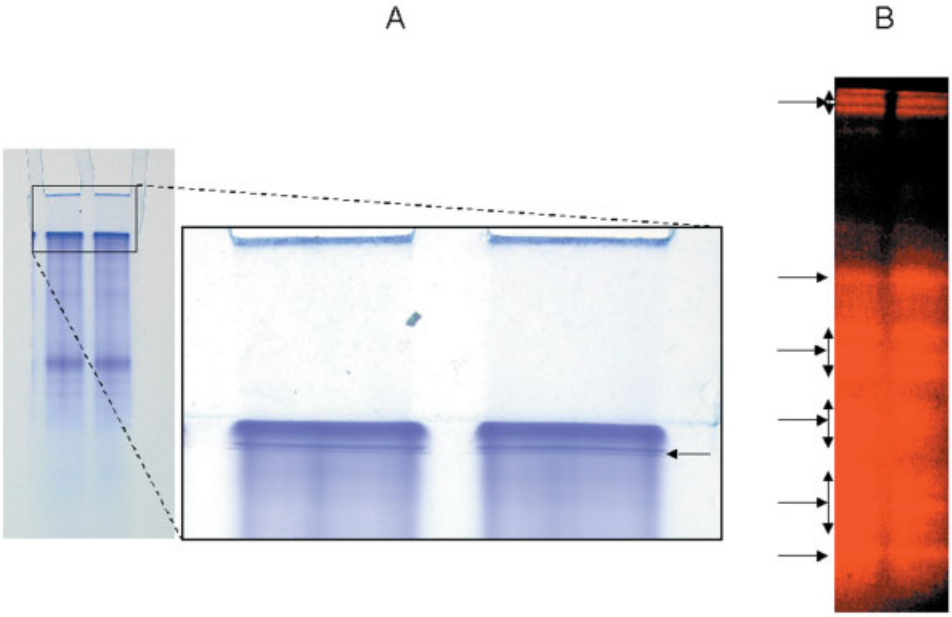

Fig. 3. **A:** Native gel PAGE of HPE proteins enriched in PG by differential precipitation with ammonium sulfate followed by SEC on Bio-Gel S-200. Proteins are revealed by their reaction with Coomassie brilliant blue. **Inset:** The arrow indicates the two bands that had barely entered the resolving gel and that were eluted from the gel to be used as the highly purified Lygus PG. **B:** PG activity gel separation and analysis of Lygus bug HPE proteins. As with the radial diffusion assays shown in Figure 1, PG activity is revealed by areas in the gel that are not stained by the ruthenium red pectin stain. However, in order to provide contrast in the photograph of the gel, black areas show stained undigested substrate and brighter red-stained bands indicate proteins with PG activity that have been separated during electrophoresis. Arrows indicate separated and clustered regions with different PG isoforms. The two bands at the top of the gel probably correspond to the bands of "purified" PG isolated from the native PAGE gel (arrow, A).

ignated "highly purified" PG. Fifteen µL of the eluted protein produced a clear zone of 123 mm^2 in the radial diffusion assay. The activity gel PG analysis of a sample of Lygus HPE that had not been subjected to ammonium sulfate precipitation and SEC fractionation (Fig. 3B) shows the full diversity of PG isoform sizes in the preparation, including two distinct high molecular weight PGs that may correspond to the two slowly migrating bands that were designated highly purified Lygus

Fig. 4. Photograph of an alfalfa inflorescence showing 4–5 straw-colored florets that failed to develop beyond the size that they were at the time of HPE injection, similar to symptoms of Lygus bug feeding damage under field conditions. Non-injected florets apical and basal to the injected florets have developed normally. Florets injected with a buffer control also developed normally.

PG and used in some of the injection tests described below.

Impact of Lygus Salivary Protein Injection on Flower Development

Under field conditions, symptoms of Lygus feeding (dry, straw-colored florets) are often expressed at the entire inflorescence level. Hence, our initial injections using pure fungal and bacterial PGs and HPE were targeted toward the center of the stem (peduncle) at the base of the developing inflorescence, which could be clearly identified under the microscope. These injections, however, gave variable results. Time-lapse video of lygus feeding on developing alfalfa inflorescences indicated that in most cases the insect fed at the bases of individual florets (approximate position of the ovary) in the developing inflorescence, rather than on the peduncle. Individual florets were difficult to align with the capillary tip due to the presence of epidermal hairs, but the microscope lighting and sample mounting technique were adjusted so that some of the florets, generally those at the base of the inflorescence, could be visualized and injected. Tests were then performed in which 2–4 basal florets per inflorescence were injected or single florets in an inflorescence were injected. Within 10 days of injection, florets either became straw-colored and withered or abscised (Fig. 4). Eleven florets each were injected with one of the three pathogen PGs or the unfractionated HPE and, in all cases, 100% of the florets exhibited the symptoms shown in Figure 4. When either salivary gland proteins or partially purified , 100% of the injected florets also developed these symptoms, whereas no symptoms were observed on any of the buffer- or BSA- injected controls (Table 1). Three alfalfa genotypes were tested over a dilution series of from 0.1 to 25% of salivary gland protein extract, and there was a clear increase in symptoms with increasing extract PG activity (i.e., total protein concentration) in a dose-dependent manner for each genotype (Fig. 5). Based on a 50% symptomatic level (SL_{50}), there were also clear genotypic differences in sensitivity, with an SL_{50} of less than 1% for M-69-10 to about 8% for UC 2705-177 (Fig. 5).

When the injector was used to target HPE to the ovary/receptacle tissue of developing cotton flowers, extensive tissue browning was observed after 3 days (Fig. 6B), while flowers injected with buffer alone (Fig. 6A), only showed browning restricted to the injection site. In most cases, the smallest flowers injected (i.e., those less than 4.5 mm in diameter at the widest point) showed no further growth following injection, and also exhibited extensive tissue browning (Fig. 6B, far right). The cumulative percentage of injected flowers that had symptoms rated as 4 (either abscission or both internal and external browning) increased with time after injection for all sizes of flowers, but symptoms were reduced as flower size increased (Fig. 7). The arrest of growth and death of small cotton flowers, whether abscission occurred or not, was similar to the response exhibited by alfalfa florets (Fig. 4). A two-way ANOVA using all injection data showed a very highly significant effect of HPE and a significant position/depth effect on the level of symptoms expressed (Table 2). However, since buffer injections gave no symptoms, and interac-

TABLE 1. Summary of Tests in Which About 6 nL Were Injected Into Individual Alfalfa Florets*

Injection treatment	No. of florets injected per inflorescence	Total no. of inflorescences tested	No. of symptomatic florets per inflorescence ± 1 SD	% Symptomatic florets
Buffer control	2–4	5	0 ± 0	0
Salivary gland extract	2–4	19	3.2 ± 1.1	(100?)
BSA control	2	6	0 ± 0	0
Buffer control	1	6	0 ± 0	0
Partially purified HPE	1	10	1 ± 0	100

*In early tests, several florets per inflorescence were injected without keeping track of the number of florets, and hence the percentage of symptomatic florets is not accurately known. In later tests, only 1 floret per inflorescence was injected. The genotype used for these studies was M-69-10.

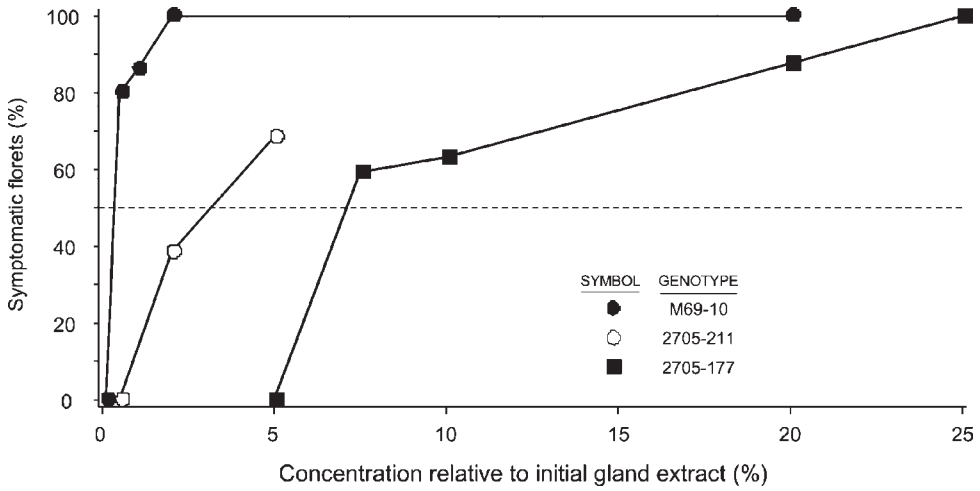

Fig. 5. Percentages of symptomatic florets following injection with 6 nL of various concentrations of HPE. The average sample size for each point is 20 florets, and the dashed horizontal line corresponds to 50% symptoms.

tions between symptom level and flower size were potentially important (Fig. 7), a further analysis of covariance, using flower size as the covariate, only for the effects of HPE injection position and depth, showed that symptoms were more severe at a depth of 2,200 μm in both staminal column and ovary/receptacle tissues than they were at a depth of 1,400 μm (staminal column only) (Table 3).

PGIP Directed Against Lygus Salivary PG in Cotton and Alfalfa Leaf Protein Extracts

The presence of alfalfa and cotton leaf proteins (PGIPs) that inhibit Lygus PG was demonstrated using the PG radial diffusion assay (Fig. 1). Proteins extracted from pear PGIP-expressing tomatoes also inhibit the Lygus PG (data not shown), suggesting that pear PGIP, selected for its ability to inhibit fungal PGs, also inhibits the insect PG. We have generated transgenic alfalfa expressing the pear PGIP gene under the control of the CaMV 35S promoter that drives constitutive expression of the pear gene (Tricoli and Teuber, personal communication). To optimize the comparison of relative Lygus bug susceptibility, the transformed line to which constitutive expression of the pear PGIP gene was to be introduced was a line selected for its low PGIP content. One of the resulting transformants (Fig. 1) shows PGIP activity that is as high as in any of the nearly 1,000 alfalfa lines we have screened (Teuber et al., 2002).

DISCUSSION

Over 30 years ago, Strong (1970) proposed that damage resulting from Lygus bug feeding on alfalfa, cotton, and other crops was primarily due to biochemical, rather than mechanical, stimuli, and principally due to the action of the insect's salivary PG. We tested these hypotheses by using a glass micro-capillary to introduce small volumes of solutions into specific areas of the floral tissues of alfalfa and cotton, presumably causing a mechanical damage comparable to that caused by insect feeding. We have shown that introduction of the substantial array of enzymes in the Lygus HPE containing little protein (generally <1 μg per injection) causes the withering and eventual abscission of alfalfa and cotton flowers, similar to the symptoms of feeding damage that are observed under field conditions. No symptoms were caused by the injection of buffer or BSA. HPE contained PG and also an array of identified and unidentified proteins, many of which might act against plant cell wall polysaccharides and other structural and non-structural substrates and could also con-

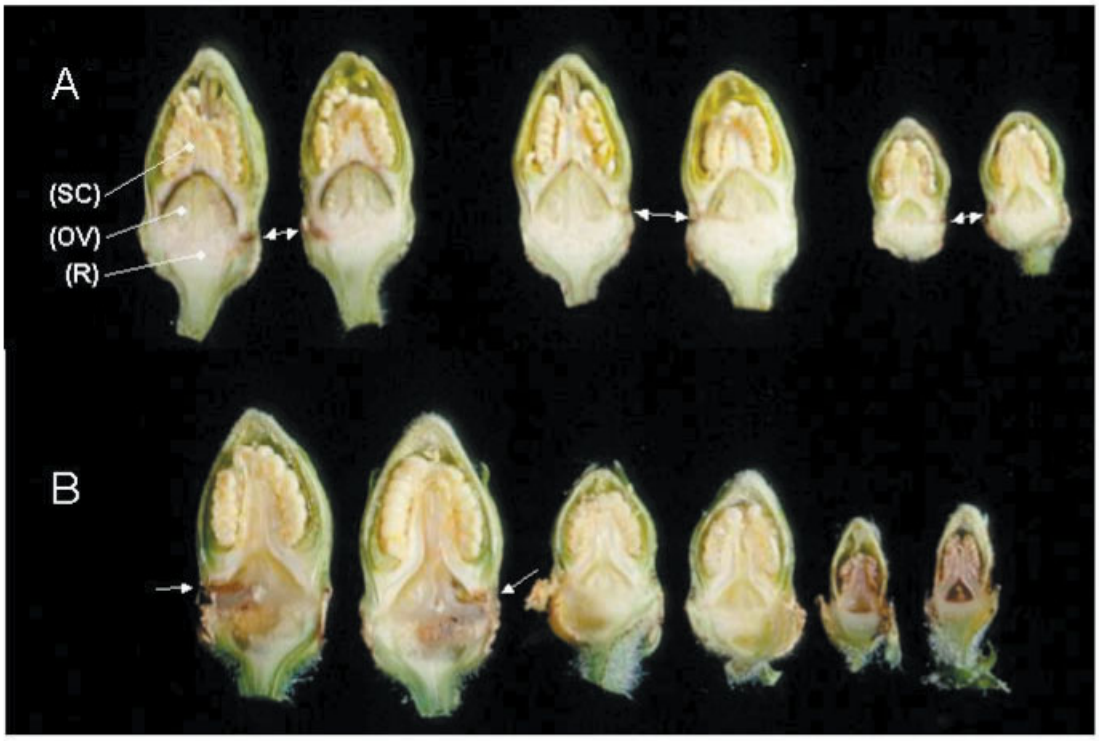

Fig. 6. Median longitudinal sections of cotton flowers (each pair shows opposite halves) injected with buffer (**A**, top row) or Lygus HPE (**B,** bottom row). Where it can bee seen, the browning indicating the site of injection is indicated by an arrow. Also indicated are the positions of the staminal column (SC), ovary (OV), and receptacle (R) tissues. The approximate range of flower sizes shown (diameter at the widest point) is 3–6 mm.

tribute to tissue damage. However, similar tissue damage was observed when extracts of excised salivary glands were used, and these extracts would be expected to contain only that assortment of proteins introduced to plant tissues in the insect's saliva during feeding. Because the same damage was also caused by injection of a highly purified, high molecular weight PG isoform purified from the

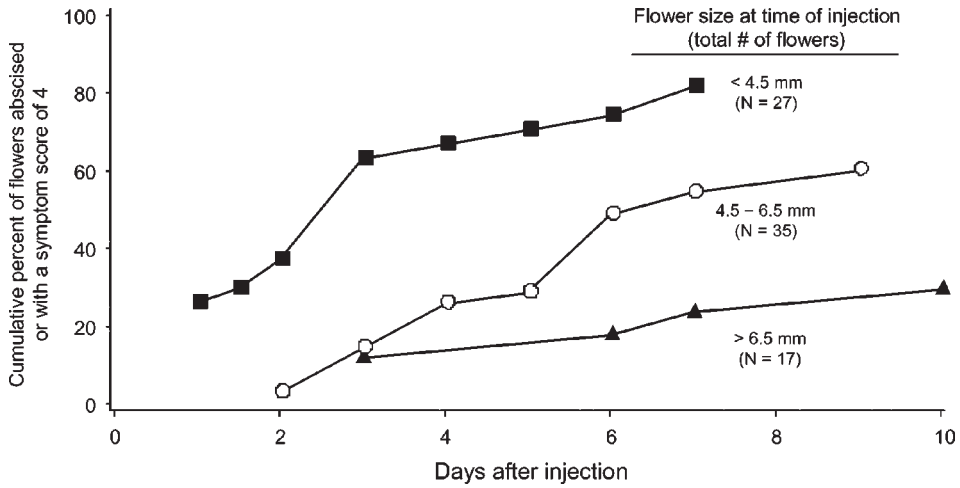

Fig. 7. Cumulative percentages of cotton flowers with a symptom score of 4 (severe internal and external browning or abscission) as a function of days post-injection, for flowers in different size categories.

TABLE 2. Two-Way ANOVA for the Effects of Material (HPE Vs. Buffer) and Injection Postion and Depth (Staminal Column at 1,400 and 2,200 μm) on the Level of Symptoms Expressed Across All Days Post-Injection That the Symptoms Were Evaluated

Source	DF	Mean square	F	Pr > F
Material	1	237.7	230.25	<0.0001
Position/Depth	2	4.5	4.35	0.0152
Error	109	1.03		

HPE, as well as pure fungal and bacterial PGs, we believe that we have largely confirmed the earlier suggestion by Strong (1970), that PG is the principal cause of Lygus feeding damage in these tissues. This is interestingly different from the other main model system for direct feeding damage by phytophagous hemipterans, hopperburn caused by *Empoasca fabae* (Harris), which results from a combination of mechanical laceration and biochemical action of saliva (Ecale and Backus, 1995). We must acknowledge, however, that the purified PG preparation we have used should not be considered to be a single protein. The staining of the protein gel (Fig. 3A) shows at least two bands in the section of acrylamide that was eluted to give the preparation. The in-gel assay of PG activity in HPE (Fig. 3B) indicates that two distinct bands of high molecular weight PG isoforms were likely present, but even this does not preclude the possibility that other proteins with undefined activities are present in the purified HPE PG used. We are continuing efforts to get several of the Lygus bug salivary PG isoforms purified for a repetition of these analyses.

Artificial introduction of solutions into plant tissues to simulate the effects of insect salivary compounds has the advantage of allowing control over the placement, quantity, and composition of materials injected, but an accurate simulation of Lygus feeding will require a more detailed understanding of the insect feeding process. For instance, the injection placements that were used in this study were based on anatomical studies of Carlson (1940), showing damage to the ovary tissue in the center of the developing alfalfa floret, and of Williams and Tugwell (2000), showing damage to the staminal column in cotton, but we were unable to find estimates of an appropriate volume of saliva to inject. We obtained a rough estimate of 300 nL for the volume of the salivary gland complex of an adult *L. hesperus* (A. Cohen, personal communication), and hence the 6-nL volume we used for alfalfa is probably a very conservative estimate of the volume of saliva available. Much smaller solution samples, on the order of 0.02 nL, have also been handled using this methodology (Shackel, 1987). The 300 nL used for cotton is a more generous estimate, but this volume was selected based on the fact that it gave the same relationship to overall flower volume as the 6 nL used for alfalfa. Unfortunately, the actual volume/activity of saliva plus the specific manner in which it is injected are unknown. How much saliva is injected with each Lygus probe, and how many probes are necessary to cause one flower abscission? Recent behavioral studies have shown that stylet penetration by Lygus nymphs on cotton squares is very active (Cline, 2000). In 2 h of electrical penetration graph monitoring of feeding, nymphs made an average of 60 probes. Mean duration per probe was only 18 sec. However, that was an average of many very short probes (~6 s each, ca. 25% of total probes) that consisted solely of salivation, plus longer probes (~2.5–6 min) with salivation and much ingestion (Cline, 2000; Cline and Backus, unreported data). It seems highly likely that during a 2-h period, a Lygus nymph could inject at least the full volume of its salivary glands. But could they secrete more, or less? This is probably dependent upon the rate of synthesis of the various salivary proteins, another important question that should be investigated.

TABLE 3. Symptoms Expressed by Cotton Flowers Injected With HPE at Different Positions on the Flower and Depths Below the Flower Surface*

Injection position	Injection depth (μm)	Total no. of flowers injected	Symptom LS mean
Staminal column	2,200	19	3.54 a
Ovary/receptacle	2,220	34	3.46 a
Staminal column	1,400	26	2.54 b
Buffer control: all positions and depths		34	(0)

*Least-squares means, adjusted for the effects of flower size as a covariate. Means followed by a different letter are significantly different at the 5% level.

At present, we can only suggest the mechanistic connection between the introduction of PG to floral tissues and the disruption of their development. Many plant tissues have been shown to make

developmental responses to the introduction of plant cell wall pectin-derived oligosaccharides (Ridley et al., 2001). Included in these responses is synthesis of the gaseous plant hormone ethylene (Campbell and Labavitch, 1991; Lurie et al., unpublished data). Ethylene's role in plant wound responses and in the regulation of organ abscission processes has been widely documented (Abeles et al., 1992). Thus the floral tissue response to PG introduction reported here could be the consequence of the enzyme's digestion of pectin polysaccharides and the subsequent perception of and response to the resulting pectin-derived oligosaccharides. A similar scenario has been proposed for aspects of plant responses to pathogens, a situation in which PG is often regarded to be a virulence factor (Collmer and Keen, 1986; ten Have et al., 1998).

In studies of plant interactions with the grey mold pathogen, *Botrytis cinerea*, work has focused on the role of plant proteins (PG-inhibiting proteins, PGIPs) that have been shown to inhibit the PGs of several, but not all, pathogens (Stotz et al., 2000) and selectively inhibit several, but not all, of the PG isoforms of *B. cinerea* (Sharrock and Labavitch, 1994). In that system, the expression of a pear fruit gene encoding a PGIP has been expressed in transgenic tomatoes and the manipulation increased tomato tolerance of the pathogen (Powell et al., 2000). An intensive screening program has also identified PGIP activity in protein extracts of alfalfa and cotton leaves. Testing of almost 1,000 individuals in the UC-2705 alfalfa population indicated that over 80% yielded proteins giving measurable inhibition of Lygus HPE PG and that those individuals containing PGIP contained a 3.5-fold range in their inhibitor content (Teuber et al., 2002). More than 1,800 cotton plant introductions (including *Gossypium barbadense* and *G. hirsutum*) have been screened for anti-Lygus PGIP. Again, most have some activity and those with activity represent a 2.5- (*G. hirsutum*) to 3.5-fold (*G. barbadense*) range in relative PGIP content (Celorio et al., 2002). PGIP activity against pathogen PGs has been reported in alfalfa and cotton (Degra et al., 1988; James and Dubery, 2001) but it is not known whether the PGIP proteins responsible for that inhibition are the ones that act against the Lygus bug PG activity.

Thus, there is a considerable germplasm base for conventional manipulation of alfalfa and cotton PGIP levels using conventional breeding approaches and the pear PGIP might prove useful for transgenic modification of PGIP content in these crops. It is likely that PGIP genes from other plant species might also encode proteins that inhibit Lygus PG. Could genetic strategies aimed at altering the amount and specific character of PGIPs in alfalfa and cotton affect their susceptibility to damage from Lygus bug and other insects with PG in their saliva in the same way that manipulation of PGIP content can influence susceptibility to fungal pathogens? While the expression of tomato resistance to grey mold can be enhanced by high transgenic expression of pear PGIP (Powell et al., 2000), PGIP is not the only factor that determines relative susceptibility to the pathogen. Guimarães et al. (2004) have concluded that several factors are involved in the grey mold resistance shown by the wild tomato relative, *Solanum lycopersicides*. Denby et al. (2004) have reported that several quantitative trait loci influence the relative susceptibility of *A. thaliana*, with one of the loci mapping close to a PGIP-encoding gene (Kliebenstein, personal communication). It is clear that different alfalfa lines incur differing degrees of developmental damage when a range of Lygus PG activity is injected (Fig. 5). However, because different germplasm lines differ in many genes, tests of the relative damage susceptibility of cultivars based on their differing PGIP contents may give ambiguous results due to the fact that several different genes, in addition to the PGIP for which we hypothesize a defensive role, could influence susceptibility to Lygus bug feeding. Thus, we have generated transgenic alfalfa with constitutive expression of the pear PGIP gene. To optimize the comparison of relative Lygus bug susceptibility, the line (clone) we transformed (UC-2525-14) was one known to be low in native PGIP content. One of the transformants (Fig. 1) shows PGIP activity that is as high as any of the individuals from the nearly 1,000

alfalfa lines we have screened (Teuber et al., 2002). This line will be used in tests like those illustrated by Figure 5 to determine the specific susceptibility to Lygus PG and, eventually, to damage caused by Lygus bug feeding.

The results presented in this report do not provide definitive data on the role of Lygus bug PG in the damage to crops that is caused by this insect. Several questions remain. Will an unequivocally pure salivary PG cause damage? If so, are all of the insect's PG isoforms equally capable of causing damage? Are other enzyme activities in the insects saliva also active in causing tissue damage? If PG is responsible for damage, what are the biochemical events that link PG introduction to developmental arrest and abscission of florets? Will the inhibition of Lygus salivary PG that has been demonstrated by proteins from alfalfa, cotton, and transgenic tomatoes in vitro be useful for mitigating insect damage in the field? In spite of these remaining questions, we feel that the data reported here provide substantial support for the hypothesis posed by Strong (1970) several decades ago, and also point the way for both conventional and molecular genetic manipulations that may significantly reduce the impact of *Lygus hesperus* as an economic crop pest.

ACKNOWLEDGMENTS

We thank Dr. Javad Habibi (Department of Entomology, University of Missouri, Columbia) for his hospitality and the training of MP Celorio in his technique for Lygus bug salivary gland isolation. The authors also acknowledge the efforts of Dr. David Tricoli (Director, UC Davis Plant Transformation Facility) for his work in producing transgenic alfalfa expressing the pear fruit PGIP gene.

LITERATURE CITED

Abeles FB, PW Morgan Jr., ME Saltveit. 1992. Ethylene in plant biology, 2nd ed. San Diego: Academic Press. 414 p.

Agblor A, Henderson HM, Madrid FJ. 1994. Characterization of alpha-amylase and polygalacturonase from Lygus spp. (Heteroptera, Miridae). Food Res Intern 27:321–326.

Agusti N, Cohen A. 2000. *Lygus hesperus* and *L. lineolaris* (Hemiptera:Miridae): the salivary and midgut enzymes. J Entomol Sci 35:176–182.

Backus E. 1988. Sensory systems and behaviours which mediate hemipteran plant feeding: a taxonomic overview. J Insect Physiol 34:151–165.

Campbell AD, Labavitch JM. 1991. Induction and regulation of ethylene biosynthesis and ripening by pectic oligomers in tomato pericarp discs. Plant Physiol 97:706–713.

Campbell DC, Dreyer DL. 1990 The role of plant matrix polysaccharides in aphid-plant interactions. In: Campbell RK, Eikenbary RD, editors. Aphid-plant genotype interactions. Amsterdam: Elsevier. p 149–170

Carlson JW. 1940. Lygus bug damage to alfalfa in relation to seed production. J Ag Res 61:791–815.

Celorio MP, Greve LC, Shackel KA, Teuber LR, Ahmadi H, Labavitch JM. 2002. Polygalacturonase role in plant damage caused by lygus bug and polygalacturonase inhibiting role in plant defense (Poster). Davis, CA: Annual Meeting of the Western Section, American Society of Plant Biologists, p 35.

Cherqui A, Tjallingii WF. 2000. Salivary proteins of aphids, a pilot study on identification, separation and immunolocalization. J Insect Physiol 4:1177–1186.

Cline AR. 2000. Stylet penetration behaviors of the western tarnished plant bug (Hemiptera: Miridae) on cotton. Entomology. Columbia, MO: University of Missouri. 150 p.

Cohen AC, Wheeler AG. 1998. Role of saliva in the highly destructive fourlined plant bug (Hemiptera: Miridae: Mirinae) Ann Appl Biol 124:405–412.

Collmer AJ, Keen NT. 1986. The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopathol 24:383–409.

Cook BJ, Clay RP, Bergmann CW, Albersheim P, Darvill AG. 1999. Fungal polygalacturonases exhibit different substrate degradation patterns and differ in their susceptibilities to polygalacturonase inhibiting proteins. Mol Plant Microbe Interact 12:703–711.

Degra L, Salvi G, Mariotti D, DeLorenzo G, Cervone F. 1988. A polygalacturonase inhibiting protein in alfalfa callus cultures. J Plant Physiol 133:364–366.

DeLorenzo G, Ferrari S. 2002. Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. Curr Opin Plant Biol 5:295–299.

Denby KJ, Kumar P, Kliebenstein DJ. 2004. Identification of *Botrytis cinerea* susceptibility loci in *Arabidopsis thaliana*. Plant J 38:473–486.

Ecale CL, Backus EA. 1995. Mechanical and salivary aspects of potato leafhopper probing in alfalfa stems. Entomol Exp Appl 77:121–132.

Guimarães RL, Chetelat RT and Stotz HU. 2004. Resistance to *Botrytis cinerea* in *Solanum lycopersicoides* is dominant in hybrids ands involves induced hyphal death. Eur J Plant Pathol 110:13–24.

Holopainen JK. 1986. Damage caused by *Lygus rugulipennis* Popp. (Heteroptera, Miridae) to *Pinus sylvestris* L. seedlings. Scand J For Res 1:343–349.

James JT, Dubery IA. 2001. Inhibition by polygalacturonase from *Verticillium dahliae* by a polygalacturonase inhibiting protein from cotton. Phytochemistry 57:149–156.

Laemlli UK, 1970. Cleavage of structural proteins during the assembly the head of bacteriophage T4. Nature 227:680–685.

Layton MB. 2000. Biology and damage of the tarnished plant bug, *Lygus lineolaris*, in cotton. Southwest Entomologist 23(Suppl):7–20.

Miles PW. 1999. Aphid saliva. Biol Rev 74:41–85

Powell ALT, van Kan J, ten Have A, Visser J, Greve LC, Bennett AB, Labavitch JM. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. MPMI 13:942–950.

Ridley BL, O'Neill MA, Mohnen D. 2001. Pectins: structure, biosynthesis, and oligogalacturonic acid signaling. Phytochemistry 57:929–967.

Shackel KA, 1987. Direct measurement of turgor and osmotic potential in individual epidermal cells. Plant Physiol 83:719–722.

Shackel KA, Matthews MA, Morrison JC. 1987. Dynamic relation between expansion and cellular turgor in growing grape (*Vitis vinifera* L.) leaves. Plant Physiol 84:1166–1171.

Sharrock KR, Labavitch JM. 1994. Polygalacturonase inhibitors of 'Bartlett' pear fruits: differential effects on *Botrytis cinerea* polygalacturobase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. Physiol Mol Plant Pathol 45:305–319.

Shen Z, Reese JC, Reeck GR. 1995. Purification and characterization of polygalacturonase from the rice weevil, *Sitophilus oryzae* (Coleoptera:Curculionidae). Insect Biochem Mol Biol 26:427–433.

Stotz HU, Bishop JG, Bergmann CW, Koch M, Albersheim P, Darvill AG, Labavitch JM. 2000. Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors. Physiol Mol Plant Pathol 56:117–130.

Strong FE, Kruitwagen EC. 1968. Polygalacturonase in the salivary apparatus of *Lygus hesperus* (Hemiptera). J Insect Physiol 14:1113–1119.

Strong, FE. 1970. Physiology of injury caused by *Lygus hesperus*. J Econ Entomol 63:808–814.

Taylor GA, Secor GA. 1988. An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. Phytopathology 78:1101–1103.

Taylor GS, Miles PW. 1994. Composition and variability of the saliva of coreids in relation to phytotoxcoses and other aspects of the salivary physiology of phytophagus heteroptera. Entomol Exp Appl 73: 265–277.

ten Have A, Mulder W, Visser J, van Kan JAL. 1998. The endopolygalacturonase gene BCPG1 is required for full virulence of *Botrytis cinerea*. MPMI 11:1009–1016.

Teuber LR, Celorio-Mancera M, Taggard KL, Greve LC, Shackel KA, Ahmadi H, Labavitch JM. 2002. Genetic variation in alfalfa for inhibition of lygus (*Lygus hesperus* L.) salivary enzymes. Sacramento, CA: Annual Meeting of the North American Alfalfa Improvement Conference.

Tjallingii WF, Hogen Esh T. 1993. Fine structure of ahpid stylet routes in plant tissue in correlation with EPG signals. Physiol Entomol 18:327– 328.

Williams L, Tugwell NP. 2000. Histological description of tarnished plant bug (Heteroptera:Miridae) feeding on small cotton floral buds. J Entomol Sci 35:187–195.

Zeng F, Cohen AC. 2001. Induction of elastase in a zoophytophagus heteropteren, *Lygus hesperus* (Hemiptera:Miridae). Ann Entomol Soc Am 94 146–151.